

Comparative study of methodologies to determine the antioxidant capacity of Al-toxified blueberry amended with calcium sulfate

C. Meriño-Gergichevich¹, G. Ondrasek², M. Zovko², D. Šamec³, M. Alberdi^{1,4}, M. Reyes-Díaz^{1,4*}

¹Center of Plant, Soil Interaction and Natural Resources Biotechnology, Scientific and Technological Bioresource Nucleus (BIOREN-UFRO), Universidad de La Frontera, Temuco, Chile. ²Department of Soil Amelioration, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia. ³Department of Molecular Biology, Institute Ruđer Bošković, Zagreb, Croatia. ⁴Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Temuco, Chile. *Corresponding author: marjorie.reyes@ufrofrontera.cl

Abstract

Blueberry (*Vaccinium corymbosum* L.) is well adapted to acidic soils where aluminum (Al^{3+}) can be linked to oxidative stress and antioxidant capacity (AC). Calcium sulfate (CaSO_4) is used to alleviate Al^{3+} because it does not alter soil acidity. However, the role of Ca addition in AC, based on a single electron transfer reaction (SET), remains unknown. The aim was to evaluate the AC using SET methodologies, i.e., 2,2-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-Azinobis-bis (3 ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing-antioxidant power (FRAP). Blueberry cultivars Elliot and Jersey were grown and exposed to the following treatments for 15 days: Control (nutrient solution alone, pH 4.5); control+Al (Al); control+Al+Ca (Al+Ca) and control+Ca (Ca). The Ca and Al concentrations, total phenol (TP) content and radical scavenging activity (RSA) were evaluated at 7 and 15 days. The Al+Ca and Ca treatments increased the Ca concentration in the leaves (22%) and roots (40%) of both cultivars compared with the control. The Ca in the tissues varied with cultivar and time. After 15 days, increases in TP, DPPH, ABTS and FRAP were detected. The cultivars showed positive relationships between the TP and AC in the leaves and roots. Thus, CaSO_4 is an important tool to improve the AC in Al-stressed fruit crops grown in acid soils.

Keywords: Aluminum, blueberry, calcium, SET assay

1. Introduction

Great interest has been shown in highbush blueberry due to its high antioxidant capacity compared with other fruits (Howard *et al.*, 2003; Dragović-Uzelac *et al.*, 2010), which provides interesting potential benefits for human health (Heinonen, 2007). This capacity is attributed mainly to antioxidant phenolic compounds with important functions, such as free radical scavengers, hydrogen-donating compounds, singlet oxygen quenchers and/or metal ion chelators (Howard *et al.*, 2003). The antioxidant capacity (AC) is associated with overcoming stresses caused by environmental factors, which may trigger an overproduction of reactive oxygen species (ROS) in plant cells (Yamamoto *et al.*, 2002; Ma *et al.*, 2007). Several studies have reported that enhanced AC is involved in the mitigation of oxidative stress in crop plants, improving their performance under the influence of this stressor. The antioxidant features depend on several factors such as cultivar, growing season, location and soil properties (Howard *et al.*, 2003; Dragović-Uzelac *et al.*, 2010).

Highbush blueberry is commonly cultivated in soils with a pH between 4.0 and 5.5; however, it is well known that in acidic soils, high amounts of toxic Al^{3+} can be released into the soil solution, damaging the roots and retarding shoot growth (Reyes-Díaz *et al.*, 2011). This cation may be associated with ROS production, which induces oxidative stress in cell organelles (Yamamoto *et al.*, 2002; Ma *et al.*, 2007). To reduce Al^{3+} levels in the soil and reverse its harmful effect on plants, a common agricultural practice is to add a calcium amendment in the form of calcium sulfate ($CaSO_4$) (Takahashi *et al.*, 2006). For plant species well adapted to acidity, such as blueberry, $CaSO_4$ represents an optimal alternative because it does not alter soil pH (Meriño-Gergichevich *et al.*, 2010; Reyes-Díaz *et al.*, 2011). The effect of $CaSO_4$ on

biochemical features such as the antioxidant capacity of blueberry has been poorly studied. Reyes-Díaz *et al.*, (2011) reported increases in nutrient content (especially Ca) and reduction in Al concentrations in blueberry cultivars after the addition of $CaSO_4$; a differential increase in the radical scavenging activity (RSA) in cultivars was also observed, concomitant with an increase in the Al concentration in the nutrient solution (100 and 200 μM).

Several analytical techniques have been used to estimate the antioxidant capacity of vegetables. Antioxidant capacity determination using 2,2-diphenyl-1-picrylhydrazyl (DPPH), for example, is based on a single electron transfer (SET) reaction involving the redox reaction with the oxidant as an indicator of the reaction end point (Ban Lee Tan and Yan Lim, 2015; Georgieva and Mihaylova, 2015). Other SET reactions are used for RSA determination: i) ferric reducing ability of plasma (FRAP), where a ferric salt ($FeCl_3$) is used as an oxidant (Benzie and Strain, 1996); ii) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), where $ABTS^+$ is the radical cation used for the assay (Re *et al.*, 1999); and iii) The Folin-Ciocalteu method or total phenolics assay (Singleton and Rossi, 1965). However, these methodologies have shown different results for the same species and growing conditions. The aim of this study was to compare different methods for determining the antioxidant capacity of two blueberry cultivars exposed to Al^{3+} and ameliorated with $CaSO_4$ at two evaluation times.

2. Material and Methods

2.1. Plant material, growth conditions and treatments

A controlled experiment was carried out in the growth chamber at the Faculty of Agriculture, University

of Zagreb, Croatia. One-year-old plants of two blueberry cultivars (Elliot and Jersey), grown in a peat substrate, were used. The roots were carefully cleaned and washed using plenty of deionized water ($<1 \mu\text{S}$). The plants were then placed into plastic pots (two plants per pot) filled with 2 L of continuously aerated and modified Hoagland's nutrient solution (Hoagland and Arnon, 1950) for conditioning during a 15-day period. After conditioning, the plants were grown in Hoagland's nutrient solution containing 5 mM CaSO_4 (Ca) and/or 100 μM AlCl_3 (Al) for 15 days. The treatments were: i) nutrient solution alone (Control); ii) Control+Al; iii) Control+Al+Ca, and iv) Control+Ca. The pH was adjusted daily to 4.5 using 0.1 M HCl and was measured using a high-accuracy portable pH meter (Milwaukee Instrument, Inc., Model SM 102, Rocky Mount, NC 27804, USA). The dissolved oxygen content in the nutrient solutions was also recorded on a daily basis using a portable meter (Mettler Toledo, SevenGo Pro). The growth chamber conditions were: 25/20 °C day/night air temperature, 16/8 h light/dark regime, relative air humidity 70%, luminescence (Lux) 5,000 lm m^{-2} and the oxygen concentration in the nutrient solution was $7.6 \pm 0.5 \text{ mg L}^{-1}$. On days 7 and 15 day of the experiment, the leaves and roots were harvested: one half was used for chemical (inorganic) analysis and the other half was stored at -30°C for biochemical analysis.

2.2. Chemical analyses

The leaves and roots of the plants were oven dried (for 24 h at 65°C in a Memmert model 410, Schwabach, Germany) and then ground in a mill. The dried and ground samples were extracted with concentrated hydrochloric and nitric acids as well as hydrogen peroxide using the microwave technique in a CEM MARS Xpress microwave unit (CEM, Matthews, N.C.) for Ca, S and Al determination using inductively

coupled plasma optical emission spectrometry (ICP-OES using a MPX AX; Varian, Perth, WA).

2.3. Antioxidant capacity determination

The antioxidant capacity of fresh leaf and root tissue was determined. The leaves were freeze-dried using a Lyovac GT 2 (Steris GmbH, Germany). The dried material (30 mg) from the plants was frozen in liquid nitrogen (-196°C), powdered in a mortar, and homogenized with 1 mL of 80% methanol (v/v). The extracts were incubated in an ultrasonic bath for 15 min, shaken for 2 h on a rotation homogenizer at 15 rpm, and then centrifuged at 10,000 g for 15 min (Eppendorf centrifuge 5451C, Germany). The supernatant was recovered and used for analysis.

2.3.1. Total phenol concentrations

Total phenols (TP) were determined using the Folin-Ciocalteu reagent following the method of Slinkard and Singleton (1997). The absorbance of the samples was measured using a UV-VIS spectrophotometer at 765 nm. The TP concentration was expressed as μg gallic acid equivalent (GAE) per g^{-1} DW.

2.3.2. DPPH radical scavenging analyses

The free radical scavenging capacity of the leaves and roots was determined according to the procedure using the stable DPPH radical (Brand-Williams *et al.*, 1995).

2.3.3. ABTS $^{\cdot+}$ radical scavenging capacity

The radical scavenging capacity of tissue extracts was evaluated using the ABTS method according to Re *et al.* (1999). This method is based on the ability of antioxidant molecules to decolorize the radical

cation ABTS^{•+}, a blue-green chromophore with characteristic absorption at 734 nm.

2.3.4. FRAP assay

The FRAP assay was conducted according to Benzie and Strain (1996). This method is based on an increase in the absorbance at 593 nm due to the formation of tri-pyridyl-S-triazine complexes with Fe²⁺ [TPTZ-Fe (II)] in the presence of a reducing agent.

2.4. Experimental design and statistical analyses

The experimental design corresponded to a factorial split-plot with six replicates. A two-way analysis of variance (ANOVA) test was used, with cultivars and treatment as the factors. All data passed the normality and equal variance tests after the Kolmogorov-Smirnov test. For the comparison of values with significant differences, a Tukey test was conducted at a significance level of $P \leq 0.05$. All statistical analyses were performed using Sigma Stat 3.1 (SPSS® Inc., Chicago, IL, USA).

3. Results

3.1. Calcium and aluminum concentration in tissues

The Ca treatment significantly increased the Ca concentration in the leaves of both cultivars (up to 40%) at 7 and 15 days in comparison with the control, whereas in plants subjected to the Al+Ca treatment, an increase in the Ca concentration was observed in the Elliot (30%) and Jersey (21%) cultivars for the same periods ($P \leq 0.05$; Table 1). The S concentration in the leaves of Elliot was 30% and 35% higher in the Al+Ca and Ca treatments, respectively, compared with the control at 7 days (data not shown). In both

cultivars, the highest foliar Al concentration in comparison to the control was observed in the Al and Al+Ca treatments at 15 days (45%) ($P \leq 0.001$; Table 1). In Jersey roots, the Ca treatment increased the Ca concentration by up to 22% in comparison with the control at 15 days ($P \leq 0.05$). However, no significant increase in the amount of Ca was observed in comparison with the control in both cultivars exposed to the combined treatment (Al+Ca). Nonetheless, in the Al treatment, a statistically significant decrease in the Ca concentration was found in both cultivars compared with the control at 7 and 15 days (Table 1). The highest increase in the Al concentration in the roots of both cultivars was observed in the Al treatment followed by Al+Ca ($P \leq 0.001$; Table 1). The S concentration in the roots of Elliot subjected to Al+Ca did not increase significantly at either time period in comparison with the control (data not shown).

3.2. Total phenols

The TPs in Elliot leaves were 70% lower in all treatments in comparison with the control at 7 days ($P \leq 0.05$; Figure 1). By contrast, Jersey leaves exhibited up to a two fold increase in TPs in the Al and Al+Ca treatments compared with the control ($P \leq 0.05$). After 15 days of treatment application, a strong increase in TPs was observed in the Al+Ca treatment in the leaves of both cultivars in comparison with the control ($P \leq 0.05$). In the roots, the TPs exhibited a ~20% increase in Elliot grown with Al and Al+Ca compared with the control at 7 days, whereas this increase in Jersey plants was found only in the Al+Ca treatment ($P = 0.028$). After 15 days of treatment application, the TPs in Elliot roots increased significantly (43%; $P \leq 0.001$) with Al treatment; however, the TPs in Jersey roots decreased by 31% in comparison with the control ($P = 0.008$).

Table 1. The effect of Al and CaSO₄ treatments on the Ca and Al content (mg g⁻¹ DW) in the leaves and roots of two blueberry cultivars at two evaluation times. Each value represents the mean of six replicates. Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) between treatments for the same cultivar and exposure time.

Organ	Content		Elliot				Jersey			
	mg g ⁻¹ DW	Control	Al	Al+Ca	Ca	Control	Al	Al+Ca	Ca	
1 st sampling										
Leaves	Ca	6.55± 0.38b	7.14±0.91b	7.18±0.24b	8.66±0.17a	5.94±0.16b	5.28±0.26c	6.50±0.28b	9.28±0.40a	
	Al	0.012±0.0003b	0.016±0.0002a	0.019±0.0001a	0.013±0.0002b	0.010±0.0007a	0.011±0.0002a	0.011±0.0003a	0.010±0.0003a	
2 nd sampling										
Leaves	Ca	7.15±0.14c	8.32±0.20c	10.7±0.23b	12.14±0.15a	5.94±0.16c	4.48±0.27d	7.56±0.26b	9.30±0.40a	
	Al	0.012±0.0003b	0.022±0.0002a	0.019±0.0001a	0.012±0.0003b	0.011±0.0007b	0.015±0.0004a	0.017±0.0008a	0.011±0.0002b	
1 st sampling										
Roots	Ca	7.00±0.16ab	3.95±0.28c	6.47±0.26b	7.49±0.22a	6.46±0.32a	3.50±0.23b	6.50±0.29a	5.82±0.15a	
	Al	0.16±0.02c	1.12±0.04a	0.72±0.02b	0.16±0.005c	0.16±0.002c	0.95±0.05a	0.64±0.09b	0.13±0.012c	
2 nd sampling										
Roots	Ca	6.96±0.16ab	3.46±0.09c	6.22±0.24b	7.17±0.28a	6.44±0.32b	4.56±0.29c	4.48±0.15c	8.35±0.36a	
	Al	0.16±0.02c	0.71±0.06a	0.46±0.02b	0.15±0.01c	0.15±0.002b	0.62±0.05a	0.63±0.09a	0.12±0.012b	

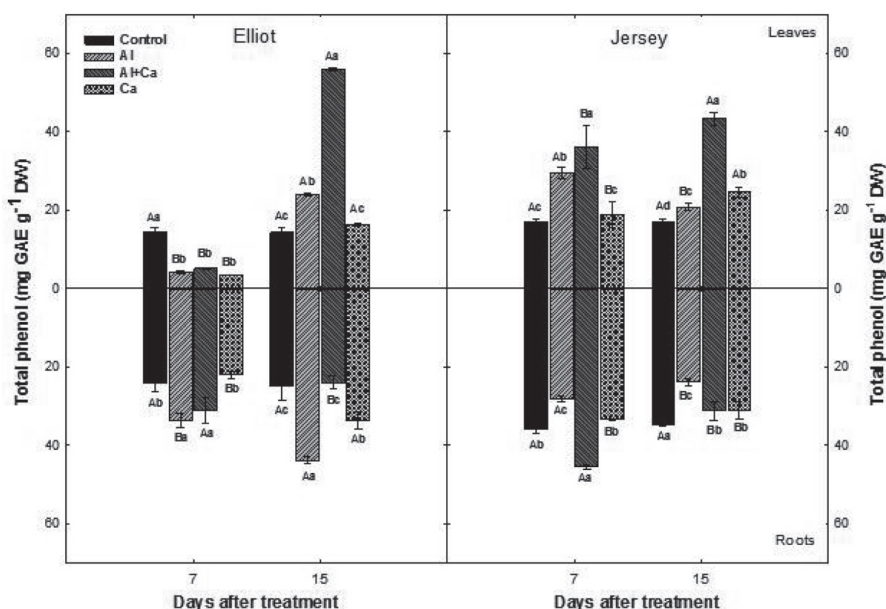


Figure 1. The effect of Al and CaSO₄ treatments on changes in the total phenols in the leaves and roots of two blueberry cultivars during the 1st (7 days after treatment) and 2nd (15 days after treatment) sampling periods. Each value represents the mean of six replicates (\pm S.E.). Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) between treatments for the same cultivar and exposure time. Different uppercase letters show differences ($P \leq 0.05$) between exposure times for the same cultivar and treatment.

3.3. Antioxidant capacity and reducing power

The total antioxidant capacity (DPPH and ABTS assays) and reducing power (FRAP assay) of the two highbush blueberry cultivars are shown in Figures 2, 3 and 4, respectively. After 7 days of treatment application, DPPH, ABTS and FRAP methods showed a reduction in the antioxidant capacity of the leaves of Elliot, whereas Jersey exhibited an increase in antioxidant capacity and reducing power in response to the Al and Al+Ca treatments in comparison with the control ($P \leq 0.001$; Figure 2). According to the results obtained at 15 days, Elliot showed an increase (approximately two fold) in

antioxidant capacity in the Ca and Al+Ca treatments compared with the control ($P \leq 0.001$).

In comparison with the control, an increase in the foliar antioxidant capacity, measured using the ABTS method, was observed at 15 days in Elliot plants subjected to Al (2-fold) and Al+Ca (3.8-fold) treatments ($P \leq 0.001$). An increase in the antioxidant capacity was detected in Jersey leaves at 7 days, which was only maintained through to 15 days in the Al+Ca-treated plants. The ABTS assay did not show any significant difference between the applied treatments (Ca and Al) and the control with respect to Elliot roots; however, in Jersey, the same parameter was significantly lower in plants treated with Al (~50%) compared with the control (Figure 3).

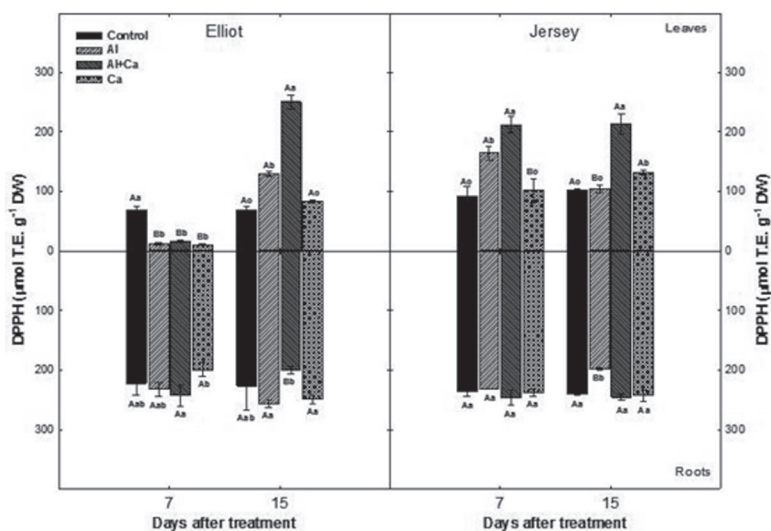


Figure 2. The effect of Al and CaSO_4 treatments on the antioxidant capacity of the leaves and roots of two blueberry cultivars measured using DPPH methodology during the 1st (7 days after treatment) and 2nd (15 days after treatment) sampling periods. Each value represents the mean of six replicates (\pm S.E.). Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) between treatments for the same cultivar and exposure time. Different uppercase letters show differences ($P \leq 0.05$) between exposure times for the same cultivar and treatment.

Similar to the DPPH and ABTS radical scavenging in the leaves at 7 days, decreased FRAP values were observed in Al-, Al+Ca- and Ca-treated Elliot plants ($P \leq 0.05$), whereas in the same treatments, Jersey showed higher FRAP values than the control ($P \leq 0.001$; Figure 4). The highest FRAP value in the leaves was found in the plants subjected to the Al+Ca treatment, i.e., 4.5- and 2.3-fold increases compared with the control in Elliot and Jersey,

respectively, at 15 days ($P \leq 0.001$; Figure 4). The FRAP values in the roots of Elliot increased significantly with the addition of Al (by 34% and 86% at 7 and 15 days, respectively) relative to the control. An increase of 57% in the FRAP value was found in Elliot roots subjected to Ca compared the control at 15 days ($P \leq 0.05$), while in Jersey, no changes were observed in the FRAP value during either time period (Figure 4).

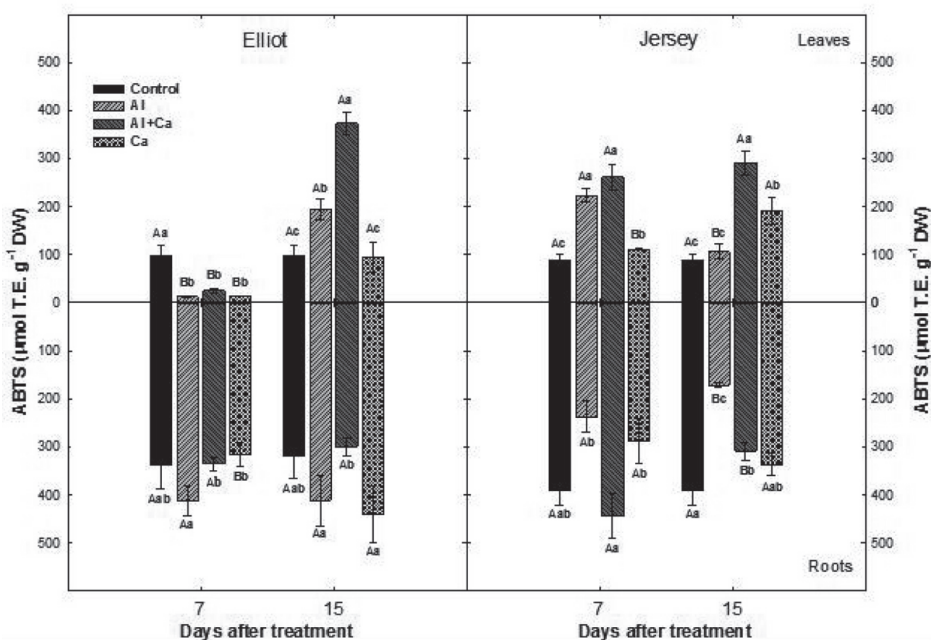


Figure 3. The effect of Al and CaSO_4 treatments on the antioxidant capacity of the leaves and roots of two blueberry cultivars measured using ABTS methodology during the 1st (7 days after treatment) and 2nd (15 days after treatment) sampling periods. Each value represents the mean of six replicates (\pm S.E.). Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) between treatments for the same cultivar and exposure time. Different uppercase letters show differences ($P \leq 0.05$) between exposure times for the same cultivar and treatment.

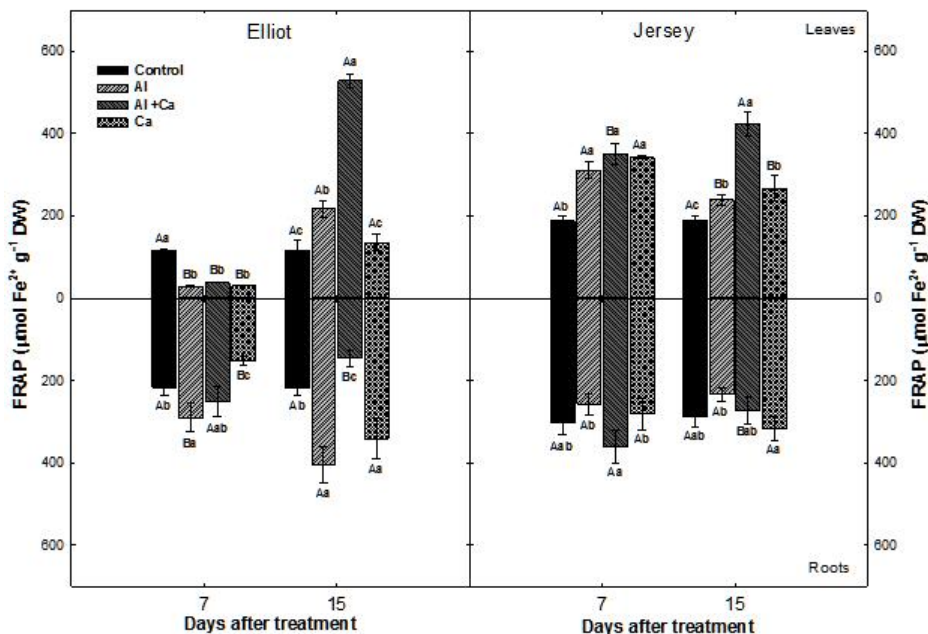


Figure 4. The effect of Al and CaSO_4 treatments on the antioxidant capacity of the leaves and roots of two blueberry cultivars measured using FRAP methodology during the 1st (7 days after treatment) and 2nd (15 days after treatment) sampling periods. Each value represents the mean of six replicates (\pm S.E.). Different lowercase letters indicate statistically significant differences ($P \leq 0.05$) between treatments for the same cultivar and exposure time. Different uppercase letters show differences ($P \leq 0.05$) between exposure times for the same cultivar and treatment.

4. Discussion

It has been reported that under acidic ($\text{pH} \leq 5.5$) conditions, significant correlations between low pH and high Al^{3+} (at micromolar concentrations) exhibit phytotoxic effects in many cultivated plants (Foy, 1998). In blueberry, which is well adapted to acidic soils with generally low Ca levels, Al^{3+} can exert a considerable negative influence on biochemical and physiological parameters, as well as plant vigor. This can lead to dieback or death, particularly in young plants, due to the accumulation of Al in tissues (Reyes Díaz *et al.*, 2010). In our study, a reduction

in Al uptake by roots was observed in the Al+Ca treatment in comparison with the plants subjected to Al alone (Table 1). The foliar Al contents (0.01 – $0.02 \text{ mg g}^{-1} \text{ DW}$) in the plants exposed to the Al+Ca treatment were lower than the range of 0.08 and $0.15 \text{ mg g}^{-1} \text{ DW}$ reported by Reyes-Díaz *et al.* (2011) for blueberry. Based on the negative correlations found between the Ca and Al concentrations in Elliot roots at 7 days ($r = -0.71$, $P = 0.009$) and 15 days ($r = -0.70$, $P = 0.01$), the reduction in the Al concentration would indicate mainly that competitive activity between calcium and aluminum in the roots was the result of the ameliorative role of CaSO_4 . Yang *et al.* (1996)

reported that in plants of the blueberry cultivar Elliot, Ca uptake was negatively affected by influx inhibition and by the blocking of Ca-specific ion channels in root cells due to its competition with Al for essential binding sites in the root apoplast. Furthermore, Al can be excluded from roots by the formation of an outer sphere complex with SO_4^{2-} (Alva *et al.*, 1991). Similar correlations were not found for Jersey, however, suggesting that genotypes within species can differ in their ability to tolerate or resist Al^{3+} by external or internal mechanisms (Foy, 1988). Although CaSO_4 soil amendment has been used as a Ca source and to ameliorate the toxic effects of Al^{3+} in acidic environments (Takahashi *et al.*, 2006), the effect of its application to berry plants has scarcely been reported, considering the multiple roles of Ca nutrition in response to environmental stress such as antioxidant performance. The control plants of the two cultivars evaluated showed similar Ca concentrations in the leaves and roots (6–7 mg g^{-1} DW), whereas the CaSO_4 -treated plants had higher Ca concentration (up to ~12 mg g^{-1} DW in the leaves of Elliot) than those reported previously for blueberries by Starast *et al.* (2007) who considered that healthy bushes could contain 3 to 8 mg g^{-1} DW of foliar Ca.

It has been reported in the literature that Al^{3+} triggers oxidative stress by increasing ROS generation in cell organelles, which activates enzymatic and non-enzymatic antioxidant responses (Yamamoto *et al.*, 2002). In particular, phenolics have an optimal chemical structure for radical scavenging activity due to their considerable ability to act as hydrogen or electron donors and to chelate transitional ion metals (Huang *et al.*, 2005; Oroian and Escriche, 2015). The biosynthesis of phenol compounds is considered to be responsible for the antioxidant capacity of blueberry (Piljac-Žegarac *et al.*, 2009) and their concentration can be linked to the availability of nutrients such as

N, P and Ca (Steward *et al.*, 2001). Increased TPs in Jersey leaves at 7 days, especially in the Al+Ca treatment, suggest that phenol synthesis is stimulated by Ca content ($r = 0.70$, $P = 0.01$) (Table 2). In Elliot, however, a negative correlation was found for the same period ($r = -0.73$; $P \leq 0.01$). Heinonen (2007) reviewed the genetic and environmental factors that could affect phenolic composition in blueberry plant tissues. In the roots of Elliot (at 7 and 15 days) and Jersey (at 7 days), TP was negatively correlated with the Ca content (Table 2). In experiments carried out on blueberry plants supplemented with Ca and boron (B) fertilizers, Eichholz *et al.* (2011) and Howard *et al.* (2003) suggested that excessive Ca content and varietal factors could be related to polyphenol synthesis. Wang and Shao (2006) indicated that oxidative stress and antioxidant systems are linked with Ca^{2+} and calmodulin proteins, which improve the ability of the plant to tolerate stress and therefore enhance the activity of their antioxidant systems. On the other hand, the Al treatment in our study was linked to leaf TPs in Jersey at 15 days ($r = 0.64$; $P = 0.02$) (Table 2). Root TPs were highly correlated with Al in Elliot and Jersey at 7 days ($r = 0.91$; $P \leq 0.001$ and $r = 0.83$; $P \leq 0.001$, respectively) (Table 2). However, at 15 days, this relationship was not observed in Elliot, while it was negative in Jersey ($r = -0.60$; $P = 0.04$), indicating the activation of other resistance mechanisms, including antioxidant enzymes.

The strong relationship between the phenolic content and antioxidant capacity in blueberry has been well documented (Howard *et al.*, 2003). Our findings show strong positive correlations between the TP and RSA measured in the leaves and roots using the DPPH and ABTS methodologies (Table 3), similar to the reports for leaves of black currant (*Ribes nigrum* L.; Tabart *et al.*, 2007), Jerusalem artichoke (*Helianthus tuberosus* L.; Yuan *et al.*, 2012), blueberry (Pervin *et al.*, 2013)

and *Origanum vulgare* leaves (Majeed *et al.*, 2015). The leaves of the two cultivars studied here exhibited greater increases in antioxidant activity in the Al+Ca treatment compared with the control, particularly at 15 days, based on the DPPH and ABTS methodologies ($P \leq 0.05$). However, Elliot did not show high DPPH or ABTS antioxidant capacity in the leaves at 7 days, suggesting that some other mechanisms (e.g., enzymatic antioxidants) were activated to prevent the harmful effects of Al^{3+} . A previous report from Reyes-Díaz *et al.* (2011) documented higher antioxidant activity (up to 100% compared with the control) in the leaves of blueberry subjected to 100 μM Al plus 5 mM CaSO_4 for 15 days, measured using DPPH. Pervin *et al.* (2013) recently observed that blueberry leaf extract showed higher scavenging properties when assayed using ABTS compared with DPPH radicals in comparison with a standard (ascorbic acid). There is very little information on comparative studies of the root-scavenging capacity of blueberry grown in the presence of phytotoxic Al^{3+} with the application of CaSO_4 to ameliorate the deleterious effects of this cation. In the roots of the blueberry cultivars Legacy and Bluegold, treatment with 100 μM Al increased the antioxidant activity (measured using DPPH) between 30-100% compared with untreated plants after 7 days (Reyes-Díaz *et al.*, 2010). In contrast, the scavenging capacity of Legacy was only ~30% higher than the control after 14 days. In our experiment, Jersey plants grown in 100 μM Al showed lower ABTS (~40%) in the roots at 7 days, which increased to 60% at 15 days relative to the control. Nonetheless, the DPPH methodology only showed a minimal reduction in the RSA in the 100 μM Al treatment at 15 days. Interestingly, we found a significant positive (approximately $r=0.80$) relationship between the root Ca content and antioxidant activity of Jersey, but not Elliot (evaluated using DPPH and ABTS).

An interesting positive correlation was found between the Al concentration and ABTS in roots for both evaluation times and cultivars. Although the antioxidant capacities of plant extracts varied between the two assay methods, these results show that the blueberry leaves and roots from the selected cultivars presented different responses to supplemental Ca and Al under variable evaluation times.

The reducing power measured using the FRAP assay is generally linked to the presence of reducing substances. These substances donate hydrogen protons to free radicals and thereby break their chains and destabilize those (Huang *et al.*, 2005). They may thus serve as a significant indicator of antioxidant capacity in different plant species (Pervin *et al.*, 2013). In this study, the leaf FRAP in all treatments showed a lower capacity in Elliot compared with the control (Figure 4). In the roots of Elliot plants, Al toxicity significantly increased FRAP at both evaluation times and was significantly correlated with TP ($r=0.94$) and DPPH ($r=0.69$) at 7 days (Table 2). This suggests that for this cultivar, the roots possess a more pronounced antioxidant mechanism against toxic Al^{3+} . Subsequently, the correlation coefficients between FRAP and TP ($r=0.87$), DPPH ($r=0.75$) and ABTS ($r=0.65$) indicated that phenolics make a greater contribution to the reducing power (Table 3). Moreover, Jersey showed a significantly higher FRAP capacity in the leaves compared with the control at both sampling points, which was probably influenced by the vitamin C content according to Guo *et al.* (2003) and Pervin *et al.* (2013).

In conclusion, the addition of CaSO_4 to blueberry induced an increase in the Ca concentration, particularly in the leaves, and this effect varied between the two varieties. This approach can be considered in the management of highbush blueberry grown under acidic soil conditions and in the

presence of toxic Al^{3+} levels because the uptake of Al^{3+} was reduced by the application of CaSO_4 . The Ca concentration was negatively related to the total phenols in the leaves of Elliot at the first evaluation point, but not in Jersey at either sampling point. In the roots, the increased Ca concentration favored the antioxidant activity in Jersey, although this effect was not rapid. This study showed evidence for the effect of Al^{3+} on the biosynthesis of phenolic compounds and radical scavenging activity in roots, but only at 7 days. Both leaf and root phenols were correlated with the antioxidant capacity assayed

using different methodologies such as DPPH, ABTS and FRAP. The results obtained for the antioxidant assays demonstrated that these methodologies can be used to evaluate the antioxidant capacity of blueberry. However, the DPPH methodology showed the strongest relationship with the concentration of non-enzymatic antioxidant compounds in both of the organs and cultivars evaluated. Finally, this work demonstrated that CaSO_4 application could be an important tool for mitigating the harmful effect of toxic Al^{3+} by improving the antioxidant performance of fruit crops established under stressed conditions.

Table 2. Correlation coefficients indicating the relationship between foliar Ca and Al with the total phenols (TPs), antioxidant capacity (DPPH, ABTS) and reducing power (FRAP) of the leaves and roots of blueberry cultivars 7 days (1st sampling) and 15 days (2nd sampling) after Al and CaSO_4 treatment. Values are the average of six replicates.

Cultivar		7-days after treatment				15-days after treatment			
		TP	DPPH	FRAP	ABTS	TP	DPPH	FRAP	ABTS
Elliot	Leaves	Ca	-0.73**	-0.63*	0.16	0.39	0.05	0.09	0.05
		Al	0.17	0.20	0.14	0.06	0.19	0.20	0.20
	Roots	Ca	-0.81**	0.39	-0.77**	-0.76**	-0.64*	0.28	0.44
		Al	0.91***	0.70**	0.88***	0.60*	0.38	0.10	0.02
Jersey	Leaves	Ca	0.70**	-0.60*	0.08	-0.64*	0.69*	0.73**	0.71**
		Al	0.13	0.11	-0.58*	0.14	0.64*	0.60*	0.65*
	Roots	Ca	-0.62*	0.25	0.46	0.23	0.54	0.80**	0.77**
		Al	0.83***	0.49	0.88***	0.76**	-0.60*	0.46	-0.72**

Significance designated as * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 3. Correlation coefficients between the total phenolic content and antioxidant capacity (DPPH, ABTS) and reducing power (FRAP) of the leaves and roots of blueberry cultivars 7 days (1st sampling) and 15 days (2nd sampling) after Al and CaSO₄ treatment. Values are the average of six replicates.

Cultivar		7-days after treatment			15-days after treatment			
		DPPH	ABTS	FRAP	DPPH	ABTS	FRAP	
Elliot	Leaves	TP	0.91***	0.73**	0.69*	0.99***	0.96***	0.97***
	Roots	TP	0.72**	0.57	0.94***	0.78**	0.65*	0.87***
Jersey	Leaves	TP	0.97***	0.97***	0.67*	0.99***	0.92***	0.98***
	Roots	TP	0.66*	0.78**	0.83***	0.84***	0.88***	0.62*

Significance designated as * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

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